

The casein micelle - the forces contributing to its integrity

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1 Introduction

The casein micelle is remarkably stable both in fresh milks and in properly processed concentrated milk products. It is the purpose of this paper to consider the factors responsible for casein micelle stability and to present models for the structure of colloidal casein micelles.

2 Forces responsible for the stability of the casein micelle

In 1929, Linderstrøm-Lang (31), as a result of his studies on casein, postulated that the colloidal milk complex should be composed of a mixture of calcium insoluble proteins stabilized by a calcium soluble protein. The latter protein would be readily split by rennin, destabilizing the colloid and allowing coagulation to occur. As we have seen, such fractions do exist. The α_{s1} - and β -caseins are indeed calcium insoluble, while κ -casein is not only soluble in the presence of calcium ions, but is readily split by rennin. In addition, Waugh and co-workers (67, 68) have demonstrated that α_{s1} - and κ -casein complexes can be reformed from the isolated fractions, as measured by sedimentation velocity experiments. Recently, Pepper (40) has demonstrated this interaction of α_{s1} - and κ -casein by gel filtration, and has studied the concentration dependence of the interaction. The complexes formed by the interaction of the isolated α_{s1} - and κ -caseins aggregate to form simulated casein micelles upon the addition of Ca^{2+} in 0.01 M imidazole buffer, pH 6.7. As viewed by electron microscopy (5), these synthetic micelles are virtually identical with fresh milk micelles except for their increased size. The precise mechanism of formation of the natural casein micelles is as yet uncertain, although several theories have been advanced based on the study of synthetic micelles and these theories will be reviewed later. In the course

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of the discussion of casein micelle structure and formation, a brief summary of the types of bonding forces which are responsible for the stabilization of protein structure will be given.

2.1 Hydrophobic interactions

One of the most significant contributions to our understanding of protein stability was made by Kauzman (29) who elucidated the nature of hydrophobic interactions in proteins. These interactions come about because water exhibits a decreased entropy as a result of the occurrence of apolar amino acid residues within the solvent. If these apolar residues are forced out of the water and into the interior of a protein molecule, where they can interact with other apolar groups, a small quantity of stabilization energy is gained per residue transferred from the solvent. Several model systems based on the energy of transfer of amino acids from water to ethanol have been studied and yield confirmatory results (4, 58). These hydrophobic interactions are highly temperature sensitive, being minimal below 5°C and maximal at higher temperatures. In a recent review article, Klotz (30) pointed out that for proteins whose crystallographic structure is known, many apolar side-chains do exist fully or partially exposed to the solvent and therefore exhibit surface patches which are available for interactions with other protein molecules.

From the amino acid analysis of the α_{s1} -, β - and κ -casein, (20, 21, 28) it is quite apparent that large numbers of apolar residues occur in these proteins. Furthermore, from the primary structures now available (Tables 1 and 2), it is clear that these hydrophobic residues are somewhat clustered for the

Table 1. Profile of the α_{s1} -casein molecule derived from its primary structure¹.

Residues considered	Net charge ²	Charge frequency ^{2,3}	Average hydrophobicity ³
1 - 40	+3	0.25	1340
41 - 80	-22½	0.75	641
81 - 120	0	0.35	1310
121 - 160	-1	0.23	1264
161 - 199	-2½	0.14	1164

¹ Adapted from Mercier et al. (34) and Ribadeau-Dumas (personal communication).

² Some error as to assignment of these values may exist, since the exact placement of all amides is not known; serine phosphate = -2, histidine = +½.

³ Calculated as described by Bigelow (4).

Table 2. Profile of the β -casein molecule derived from its primary structure¹.

Residues considered	Net charge ²	Charge frequency ^{2,3}	Average hydrophobicity ³
1 - 43	-16	0.65	783
44 - 92	-3- $\frac{1}{2}$	0.13	1429
93 - 135	+2	0.23	1173
136 - 177	+3	0.07	1467
178 - 209	+2	0.06	1738

¹ Derived from the data of Ribadeau-Dumas (personal communication).

² Some error as to assignment of these values may exist, since the exact placement of all amides is not known; serine phosphate = -2, histidine = + $\frac{1}{2}$.

³ Calculated as described by Bigelow (4).

α_{s1} - and β -caseins, as well as for κ -casein (25). According to the calculations of Hill & Wake (25), the caseins rank among the most hydrophobic proteins of those tabulated by Bigelow (4). It is not unexpected then, that the casein micelle should be stabilized by hydrophobic bonding. Several investigators (10, 16, 47, 54) have noted that β - and κ -caseins, and α_{s1} -casein to some extent, diffuse out of the micelle at low temperatures. As one decreases the temperature, hydrophobic stabilization energy decreases, and these molecules (β - and κ -casein) are able to diffuse out of the micelle. These observations are consistent with the known primary structure of β -casein (Table 2) and κ -casein's postulated structure (25). β -casein's interactions are more temperature-dependent, which indicates that it is probably more 'soap-like' than κ -casein. While all of the authors cited above agree that β -casein, and to a lesser extent κ - and α_{s1} -caseins, can be removed from the casein micelle at 1°C, some question arises as to the exact amount released. Rose (47) reported high values for β -casein (up to 30%), while Downey & Murphy's values (16) (up to 15%) are lower. The latter workers, however, pointed out that the stage of lactation and health of the animal play a role in the amount of cold soluble casein present. All of those cited above concur that the α_{s1} -fraction does not diffuse from the micelle to as great an extent as the other two caseins.

The rare α_{s1} -A genetic variant, however, does exhibit highly temperature dependent interactions. The α_{s1} -A gene is the result of the sequential deletion of up to 13 amino acid residues (20, 34, 61) bounded by residues 13 and 27 and the majority of these deleted amino acids are apolar (61). The net result of this deletion is to bring the charged phosphorus-rich area closer to the N-terminal region, making this α_{s1} -genetic variant more β -casein-like in its

charge distribution, and the physical and solubility properties of α_{s1} -A mirror those of β -casein (62). Thus, the stability of the casein micelle is due in part to hydrophobic interactions, although some ionic bonding must occur between the α_{s1} - and κ -caseins; the α_{s1} -A deletion probably does not permit the formation of the ionic bonds characteristic of α_{s1} -casein and, as a result, micelles containing this protein are less stable to heat, cold, and processing conditions.

Dissociating agents, such as sodium dodecyl sulfate, guanidinehydrochloride, and urea, all of which are thought to act primarily on hydrophobic interactions, tend to disrupt casein micelle structure in the same fashion, as evidenced by electron microscopy (8). These solvents reduce the micelle to small subunits approaching 10 nm in diameter. The temperature-dependent properties of the hydrophobic interactions may also explain why milk can withstand moderate to high temperatures, but does not survive extremely low temperatures, such as freezing.

2.2 *Electrostatic interactions*

It has been pointed out (6, 30) that essentially all of the ionic side-chains in the proteins, whose crystallographic structure is known, are fully exposed to the solvent. Ionic bonding then, between negatively charged carboxylic acid residues and positively charged groups, contributes little to the stability of a monomeric protein. Notable exceptions to this rule may occur when an ion pair can be formed within a hydrophobic environment (58), the interactions of subunits of a protein may provide just such an environment. Physical-chemical evidence for the role of ionic bonding in subunit interactions is abundant, while crystallographic evidence is limited to hemoglobin (18) although several subunit enzymes are currently under study (23). Conversely, electrostatic interactions between carboxylate residues and divalent metal ions can impart reasonable structural stability to a protein. Calcium stabilizes staphylococcal nuclease (23) and increases the heat stability and reactivity of trypsin (53). Many metallo-enzymes derive a good deal of their stabilization from specific metal coordination complexes (15).

The role of inter- and intra-molecular ionic bonds among the α_{s1} -, β - and κ -caseins in stabilization of micelle structure is difficult to assess. Many potential sites for strong ion pair bonds within an apolar environment exist as deduced from consideration of the known sequences; and such bonds may play a role in micelle subunit interactions. Pepper et al. (42) demonstrated that carbamylation of 5 of 9 lysine residues of κ -casein abolished the ability of κ -casein to stabilize α_{s1} -casein, thus demonstrating that ionic interactions

may play a role in micelle structure. Furthermore, Hill (24) modified the arginine side-chains of the caseins and found differences in coagulation by rennin.

The estimated calcium content of milk is around 30 mM (12, 70) far above the concentrations of Ca^{2+} required to precipitate the isolated α_{s1} - and β -caseins at room temperature (62). The role of the phosphate residues in calcium binding has been investigated by the enzymatic dephosphorylation of α_{s1} -casein. Pepper & Thompson (41) and Bingham et al. (5) demonstrated that dephosphorylated α_{s1} -casein was still precipitated by calcium and showed decreased stabilization by κ -casein. The latter authors postulated that two non-phosphate calcium binding sites occur in α_{s1} -casein, and that it is the binding to these sites which induces precipitation of the dephosphorylated casein. The investigation of the κ -casein stabilized, dephosphorylated α_{s1} -casein by electron microscopy (5), showed larger but fewer micelle-like structures. In milks containing α_{s1} -A (62, 63), such large micelles are poorly solvated and less stable. Thus, the formation of micelle-like structures is not totally dependent upon the formation of calcium-phosphate bonds between caseins; however, the resulting micelles may be less stable. Indeed, removal of calcium from micelles by chelating agents such as EDTA and fluoride (8) leads to disruption of casein micelle structure as evidenced by electron microscopy.

The total number of charged groups of the casein monomers (Tables 1 and 2) reveals that in the formation of a casein micelle, not all of these ionic groups can occupy a surface position. This would indicate that either much energy is used to bury these groups or the structure is porous and available to the solvent, water. The latter proposition is borne out by the experimental evidence. Ribadeau-Dumas & Garnier (45) noted that carboxypeptidase A is able to remove, quantitatively, the carboxyl-terminal residues from the α_{s1} -, β - and κ -caseins of native micelles, demonstrating that this enzyme (mol. wt 40 000) is able to penetrate into the center of the casein micelle. Thompson et al. (62, 63) have shown that the casein micelle is a highly solvated structure with an average of 1.90 g of water per g of protein. They also noted a strong positive correlation between the degree of solvation and heat stability (63). The degree of solvation of the micelle, and hence the heat stability of the milk, hinges upon a variety of factors (43, 46, 63) not the least of which is the calcium : phosphate ratio. Increases in the calcium content of milk causes decreased heat stability, (43, 46, 65) possibly by altering the degree of solvation of the casein micelle. Thus, the micelle emerges as a highly solvated porous structure. Environments which tend to decrease solvent interaction lower the stability of the micelle, which in turn destabilizes the milk. These interactions relate back to the proposition that

the ionic residues of the individual casein monomers cannot be totally buried but must be exposed to solvent.

The better early measurements of the monomer molecular weights of the isolated casein fractions were obtained at pH 11 to 12 (33, 67). At these pH's, the positively charged lysine residues and a portion of the arginine residues have been neutralized, thus increasing the charge repulsions of the carboxyl and phosphate residues. However, prolonged exposure to high pH may produce degradation as pointed out by Noelken (36). These same effects operate in the casein micelle; as the pH of milk is brought to 11 to 12, the micelle structure is disrupted with accompanying changes in turbidity and viscosity. Presumably, exposure to high pH for long periods of time, for example, in the production of sodium caseinate, may cause degradation, and hence alter the characteristics of the product.

2.3 Hydrogen bonding secondary and tertiary structure

Many globular proteins, such as myoglobin, are stabilized by a high degree of α -helical structure (6). In addition to the fibrous proteins, the so-called β - or pleated sheet structure has been detected by X-ray crystallography in globular proteins, notable lactate dehydrogenase (23) and others (6, 18). These secondary structures are stabilized by the formation of hydrogen bonds along the polypeptide backbone. Many proteins have been shown to contain significant amounts of secondary structure, as determined by spectral methods such as circular dichroism, optical rotatory dispersion and infrared spectroscopy (64). However, in at least one case these methods have proven inaccurate in predicting the amount of secondary structure of a protein (3). Therefore, the spectral methods can provide a good estimate of the amount of secondary structure, but they are subject to error. In many cases, then, some degree of stabilization is achieved by the formation of α -helical or β -structure, but not all stable proteins contain considerable amounts of these conformations. Other bonding forces (noted above) and perhaps even 'sterically restricted' random structures may contribute significantly to the stabilization of a protein. The formation of α - and β -structures is also highly dependent upon the amino acid side-chains (proline, for example, breaks helical structures). In fact, the state of ionization of the side-chains and the solvent used play a role in the formation of α -helix (6, 18).

Spectral investigations of the isolated caseins have shown that these proteins possess little secondary structure. Herskovits (22) demonstrated by optical rotatory dispersion, using Moffit-Yang, Drude and Shechter-Blout analyses, that in aqueous solutions, neither the individual casein components (α_{s1} , β -

or κ -) nor whole sodium caseinate exhibit an appreciable degree of α -helical content. Noelken and Reibstein (37) concluded that β -casein exhibits a random coil conformation in both aqueous solution and in 6 M guanidine-HCl. Evidence has been accumulated (39, 49, 57, 69) that α_{s1} - and β -caseins are intermediate between a totally random and a globular protein in conformation. The above observed properties of the caseins are in good agreement with the high incidence of proline scattered throughout the α_{s1} - and β -caseins as derived from analyses of their sequences. Since little or no secondary structure occurs in the individual casein components, one would expect that the degree of stabilization contributed to the casein micelle by α -helix or β -structure would be quite low.

Theoretically, hydrogen bonds between ionizable side-chains accessible to the solvent, water, contribute to a limited degree to the stabilization of monomeric proteins (6, 30). These groups are already hydrogen bonded to water and the water-residue hydrogen bond must be broken, before a residue-residue can be formed. Nevertheless, once two subunits of a protein begin to interact, these surface groups may no longer be totally hydrated and hydrogen bonds could form between monomers as a result of the altered environment.

Hydrogen bonding between casein monomers in the casein micelle may occur. Subunit interactions, at present, have not been sufficiently detailed by crystallographic evidence to support or rule out these types of bonds, but some intra-chain hydrogen bonds do occur in monomeric proteins (6). It is also possible that some hydrogen bonding may occur in the self-association (49, 51) of α_{s1} -casein. Certainly, in the formation of the highly aggregated casein micelle, such bonds between the various casein components would be possible.

2.4 The role of disulfide bonds

The folding about of helical segments, pleated sheet areas and unordered structures of a polypeptide chain is referred to as tertiary structure. The tertiary structure of proteins can be locked in place by the formation of disulfide bonds between distal cysteine residues. In fact, non-identical polypeptide chains can be held together by disulfide bonding as in the case of γ -globulins. Evidence has been presented that for several proteins, the disulfide bridges do not cause the formation of secondary and tertiary structure, but tend to stabilize the preformed conformations (6). Proteins such as lysozyme and RNase with a relatively high degree of disulfide bonding are quite stable, but not all stable proteins necessarily contain disulfide bonds.

As noted above, κ -casein is the only major component of the casein protein complex which contains cystine (or cysteine). The occurrence of free sulfhydryl groups in the native casein complex has been reported by some workers (2), but not by others. Hence, the degree of disulfide cross linkages, which normally occur in the casein micelle, is difficult to estimate. Swaisgood & Brunner (55) reported that a good approximation of the minimum size of κ -casein would be a disulfide linked trimer, but for the most part, their evidence (55, 56) would indicate a greater degree of cross linking. However, Woychik et al. (71) demonstrated that reduced and alkylated κ -casein stabilized α_{s1} -casein against calcium precipitation as well as native κ -casein. It appears that while the disulfide bridges of the casein micelle may contribute to the overall stability of the casein micelles, they are neither the driving force for micelle formation nor the central feature of the formed micelle.

2.5 Colloidal calcium phosphate

The total calcium content of skim milk has been estimated to be 30 mM (12, 70), but the calcium ion content of serum, prepared by ultrafiltration or centrifugation of skim milk is only about 2.9 mM (7, 12). Specific ion electrode studies yield a value of 2.5 mM calcium (II) for skim milk (13). Thus more than 90% of the calcium content of skim milk is in some way associated with the casein micelles. Subsequent washing of the micelles removes only a small portion of the calcium and other salts. The mineral content of washed micelles, prepared by centrifugation (12), and 'primary micelles', prepared by gel filtration (7), are compared in Table 3; both methods appear to yield similar calcium and phosphate contents. The existence of this so-called 'colloidal calcium phosphate' was postulated as early as 1915 by Van Slyke & Bosworth (66), who concluded that the non-protein bound colloidal calcium phosphate was present in a 1 : 1 molar ratio which approximates di-calcium phosphate. Later workers (12, 70) have calculated that the colloidal calcium phosphate more closely resembles tri-calcium phosphate with a Ca : PO₄ molar ratio of 1 : 5. Calculation of such a ratio after subtracting casein-bound calcium is subject to inherent error. Binding studies (14) on the isolated β - and κ -caseins show a good 1 : 1 correlation between calcium ions bound and phosphate residues, while α_{s1} -casein appears to have (5, 14) one to two non-phosphate calcium binding sites. The application of these results allows the calculation of a Ca/PO₄ molar ratio from Table 3. The ratio obtained for washed micelles and micelles prepared by gel filtration are 1 : 6 and 1 : 8, respectively. The latter value differs from that calculated by Boulet et al. (7) because they assumed a 2 : 1 casein phosphate : calcium ion

Table 3. Total mineral composition of casein micelles (mM/100 g casein)¹.

	Washed micelles ² by centrifugation	Micelles by gel filtration (7)	Unwashed micelles by	
			centrifugation	gel filtration (7)
Calcium	69.6 64.1	68.9	71.0	79.0
Magnesium	4.2	3.3	4.5	6.9
Sodium	4.5			
Potassium	6.2	6.2		
Casein PO ₄	22.2 23.2	28.2		
Inorganic PO ₄	28.9 27.8	21.8	47.8	43.2
Citrate	1.6	0.0	6.2	4.7

¹ Casein N × 6.4.² Adapted from McMeekin and Groves, Fundamentals of dairy chemistry, 1st ed., Chapter 9, Table 70.

ratio. Thus, attempting an exact assignment of calcium to either the casein fraction or the colloidal calcium phosphate fraction can cause discrepancies in the calculated ratio. It must be realized that these data are average values based on average distributions of the caseins and the minerals. Not only does the mineral content and the casein distribution vary from one individual milk to another, but the various micelle fractions within a single sample are probably not of uniform composition.

It is clear from Table 3 that there are two distinct forms of ions associated with the casein micelle, an outer system perhaps in the form of a charged double layer (7) and an inner system not easily washed away. As we have noted above, the casein micelle is a highly porous, well solvated system and the occlusion of ions within this network is not unexpected; however, some actual complex formation between the colloidal calcium phosphate and the casein cannot be ruled out. If one examines the pK's of phosphoric acid, it would seem most likely that the associating species of phosphate would be (HPO₄)²⁻. Termine & Posner (59) studied the in vitro formation of calcium phosphate at pH 7.4, and concluded that an amorphous calcium phosphate phase (with a Ca/PO₄ molar ratio of 1 : 5) formed prior to the transition to crystalline apatite. In a subsequent study (60), it was shown that casein and some other macromolecules enhanced the stability of the amorphous calcium phosphate and, in fact, retarded the amorphous → crystalline transition. It would appear then that conditions should favor the formation of an amorphous-calcium phosphate-caseinate complex in milk. The exact

nature of this complex (or occlusion) is as yet undetermined though its role in casein micelle stabilization is well-documented. Pyne & McGann (44) demonstrated that the colloidal calcium phosphate content of milk decreases as the pH is lowered from 6.7 to 5.0 at 5°C. If a small sample of this pH 5.0 milk is then dialysed at 5°C against several large volumes of the original milk, the pH returns to 6.7, but the colloidal calcium phosphate is no longer present. Milk brought to essentially zero colloidal calcium phosphate concentration at pH 5, and dialysed back to 6.7 in this manner, has been termed colloidal calcium phosphate free milk (CPF-milk). In a later study, McGann & Pyne (32) investigated the properties of CPF-milk as compared to the original untreated milk. The CPF-milk is translucent as compared to ordinary milk, and has a greatly increased viscosity. Addition of Ca^{2+} up to about 1 M has little effect on normal milk at 25°C, provided the increase in pH is not compensated for. CPF-milks, however, are precipitated at added calcium ion concentrations of only 25 mM. There is no apparent difference between the CPF and normal milks with regard to the primary phase of rennin attack as measured by release of soluble nitrogen, but, interestingly, the CPF-milks are slightly more heat stable. Finally, McGann & Pyne (32) noted that, at low temperatures, β -casein is more firmly bound to rennin clotted normal milk than to rennin clotted CPF milks. Jennes et al. (26) noted a marked increase in serum or non-micellar casein, accompanied by an increased translucence as the colloidal calcium phosphate content of milk was reduced by the addition of EDTA. Rose (47) noted that while Ca^{2+} addition generally decreases the serum casein content of milk, the lowering of the pH of milk to 5.3 and the subsequent release of Ca^{2+} actually increases the serum casein content. This result led Rose (47) to speculate that the colloidal calcium phosphate aids in maintaining micelle stability. CPF-milks and normal milks were compared by Downey & Murphy (16) with respect to their elution volumes on gel chromatography (Sephadex 2B) in a synthetic milk serum. The normal casein micelles eluted at V_0 yielded a molecular weight of $>10^8$, but CPF-micelles eluted at a volume consistent with a molecular weight of about 2×10^6 . However, this result could also be explained by a marked change in shape (frictional ratio).

All of the discussion presented above indicates that the colloidal calcium phosphate is involved in maintaining the structural integrity of the casein micelle. Occlusion of amorphous apatite or possible complexation of the mineral must occur, but the exact mechanism by which stabilization is achieved is as yet unknown.

3 Casein micelle models

3.1 Basis for model construction

While it remains unclear what role, if any, the genetic variants of the caseins play in casein micelle formation, we feel that current concepts proposed for micelle formation deserve mention. For the nutritional function of caseins, it is not unusual that most of the observed casein polymorphs have no apparent deleterious effects on the micellar system. However, α_{s1} -A represents the sequential deletion of about 4% of the α_{s1} -molecule, and altered properties of milks containing this variant might be expected. Indeed α_{s1} -A milks are difficult to process and yield poor cheeses. The individual caseins have been studied in great detail; yet the precise structure and mechanism of formation of the casein micelle, is not known. Nearly as many models have been proposed as there are investigators. Let us briefly consider why the situation exists. Electron microscopy of the casein micelles of bovine milk indicates an average diameter of about 140 nm for the spherically shaped micelles. Thus, the volume occupied by a micelle would be in the order of about $1.4 \times 10^6 \text{ nm}^3$. For comparison, the β -lactoglobulin monomer occupies a volume of about 24 nm^3 . Theoretically, more than 50 000 β -lactoglobulin-like monomers could be arranged into a sphere the size of a casein micelle. Molecular weight measurements for the micelle range from 10^7 to 10^8 . A speculative calculation — $(3 \alpha_{s1} + 2 \beta + 1 \kappa)/6$ — based on an average mol. wt of 23 000 for the casein monomers, and employing only 25 000 monomers yields a micelle molecular weight of 6×10^8 . This would indicate a low density packing of the casein monomers which is consistent with the high hydrations, the random structures and the high negative charge densities of the caseins, as compared to β -lactoglobulin. It is therefore understandable that the mechanism of assembly of this aggregate of around 25 000 monomers has not been fully elucidated. For the purpose of discussion, we shall group the various proposed models into three classes.

3.2 Coat-core models

The first class of models to be discussed actually contains two diametrically opposed theories. The model proposed by Waugh and his coworkers (48, 69) is primarily based upon their studies of the Ca^{2+} solubilities of the caseins. The model, in essence, describes the formation of low weight ratio complexes of α_{s1} - and κ -casein in the absence of calcium. Upon addition of calcium ions, the α_{s1} - or β -caseins, which are represented by monomers with a charg-

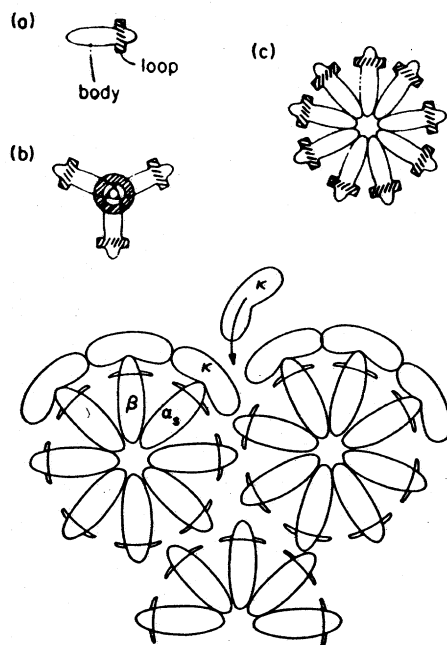


Fig. 1. Waugh's proposed model for the casein micelle: a. monomer model of α_{s1} - or β -casein with charged loop; b. a tetramer of α_{s1} -casein monomers; c. planar model of a core polymer of α_{s1} - and β -caseins. The lower portion shows how κ -casein might coat core polymers. Adapted from Rose (48).

ed phosphate containing loop, begin to aggregate to a limiting size (the caseinate core). In the presence of the low weight ratio α_{s1} - κ -complexes, precipitation of the casein is prevented by the formation of a monolayer of these low-weight α_{s1} - κ -complexes which envelops the core aggregates. This coat has the κ -casein monomers spread out on the surface and micelle size is therefore dictated by the amount of κ -casein available. In the absence of κ -casein, the α_{s1} - and β -cores agglutinate and precipitate from solution. Waugh's model, as presented in Fig. 1, has a good deal of appeal since it explains the lyophilic nature of the colloidal casein complex, as well as the ready accessibility of κ -casein to the enzyme rennin.

Parry & Carroll (38) attempted to locate this surface κ -casein proposed by Waugh by use of electron microscopy. Using ferritin-labeled anti- κ -casein immunoglobulins, they investigated the possibility of surface κ -casein and found little or no concentration of κ -casein on the surface of the casein micelles. Based on these results, and the size of the isolated κ -casein complex, Parry concluded that the κ -casein might serve as a point of nucleation, about which the calcium insoluble caseins might cluster and subsequently be stabilized by colloidal calcium phosphate (see Fig. 2). The action of rennin on the

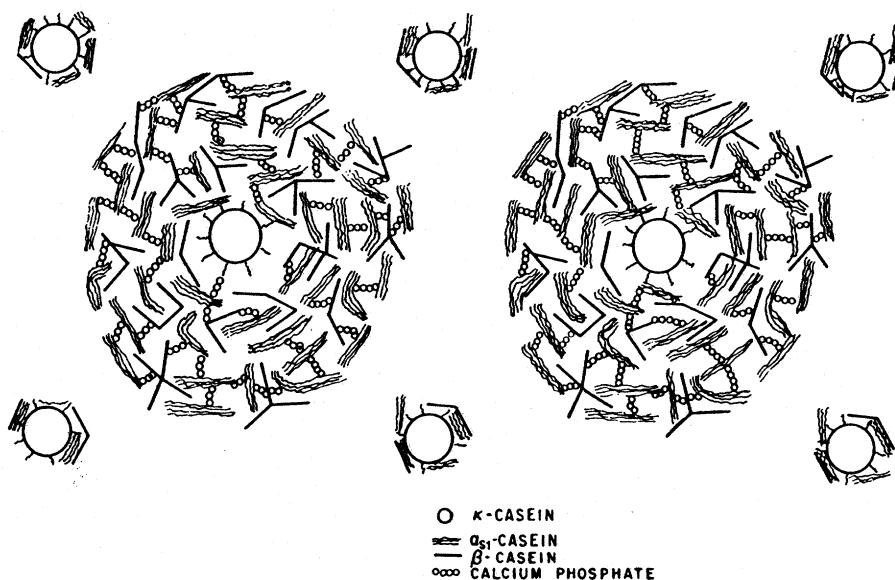


Fig. 2. Casein micelle model proposed by Parry & Carroll (38), depicting the location of κ -casein in the micelle.

micelles was accounted for by demonstrating that serum κ -casein can participate in coagulation and may be involved in the formation of bridges between micelles.

The models of Parry and Waugh both predict a non-uniform distribution of κ -casein and in a sense are based upon nucleation about a core (Parry's core = κ -casein; Waugh's core = α_{s1} -, β -calcium caseinate). It is important to note that both models predict no particular stoichiometry for the casein components and demonstrate no subunit structures composed of all three casein components. Secondly, Waugh's model does not incorporate any colloidal calcium phosphate which, as noted above, plays an important role in casein micelle structure and stability. Finally, Ashoor et al. (1) have recently demonstrated that papain, which had been cross linked by glutaraldehyde into a large insoluble polymer, caused proteolysis of all three major components of isolated casein micelles. The α_{s1} -, β - and κ -caseins were all cleaved proportionately by the enzyme super polymer. Therefore, all three components must occupy surface positions on the micelle in relatively the same proportions in which they occur in milk. This result would seem to rule out any preferential localization of κ -casein.

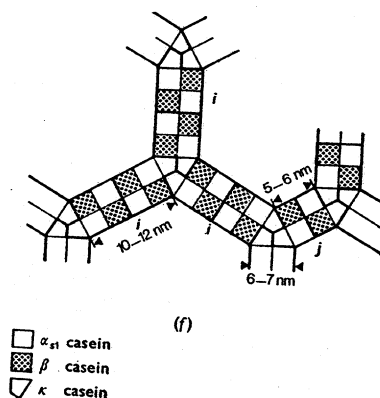


Fig. 3. Structure of the repeating unit of the casein micelle adapted from Garnier & Ribadeau-Dumas (19).

3.3 Internal structure models

The second class of models to be discussed are based upon the known properties of the isolated casein components, which in turn cause or direct the formation of the internal structure of the casein micelle.

Garnier & Ribadeau-Dumas (19) have proposed a model for the casein micelle, which places a good deal of emphasis on κ -casein as the keystone of micelle structure. Trimers of κ -casein are linked to three chains of α_{s1} - and β -casein which radiate from the κ -casein node (a Y-like structure), as shown in Fig. 3. These chains of α_{s1} - and β -casein may connect with other κ -nodes to form a loosely packed network. Garnier & Ribadeau-Dumas favor this type of network because it yields an open, porous structure and they have demonstrated that carboxypeptidase-A with a molecular weight of about 40 000 is able to remove the C-terminal amino acids of all the casein components. The model satisfies the demonstrated porosity, but places great steric restraints upon κ -casein which possesses no α -helical or other prominent secondary structures. In addition, studies by Cheeseman (11) and others indicate that while disulfide linked trimers of κ -casein do occur, the majority of the κ -casein may form aggregates of higher, as well as lower, orders. Finally, the model assigns no definite role to calcium caseinate interactions, and ignores the possibility of colloidal calcium phosphate involvement in stabilization of the micelle.

Rose used the known endothermic polymerization of β -casein as the basis for his micelle model. In this model β -casein monomers begin to self-associate into chain-like polymers to which α_{s1} -monomers become attached (Fig. 4) and κ -casein, in turn, interacts with the α_{s1} -monomers. The β -casein of the thread is directed inward, the κ - outward, but as these segments coalesce, a

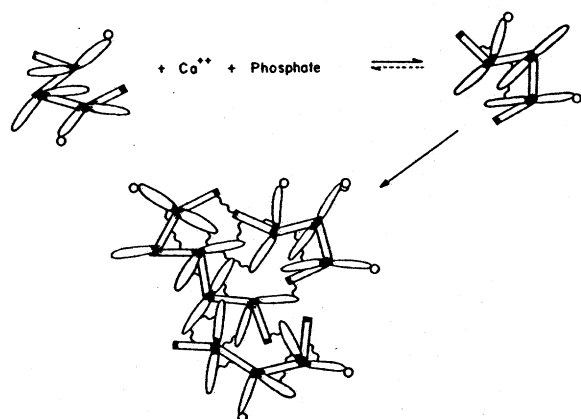


Fig. 4. Schematic representation of the formation of a small casein micelle. The rods represent β -casein, the more elliptical rods represent α_{s1} -casein and S-shaped lines depict apatite chain formation. The circles represent κ -casein. Adapted from Rose (48).

small amount of κ -casein is inevitably placed in an internal position. As the micelle is formed, colloidal calcium phosphate is incorporated into the network as a stabilizing agent. The model is appealing in that it accounts for the occurrence of some overall stoichiometry of the various casein components, while demonstrating the role of colloidal calcium phosphate in micelle stabilization. The choice of β -casein as the basis for micelle formation is, however, questionable since Waugh et al. (69) have shown that the α_{s1} - and β -caseins tend to form mixed polymers randomly; secondly, β -casein is quite structureless in solution and, finally, synthetic micelles can be formed from simple α_{s1} - and κ -casein complexes in the absence of β -casein.

3.4 Subunit models

The final class of models to be discussed are those which propose subunit structure for the casein micelle. Shimmin & Hill (52) proposed such a model based upon their study of ultra-thin cross-sections of embedded casein micelles by electron microscopy. They predicted a diameter of 10 nm for the subunits of the casein micelle.

Morr (35) studied the disruption of casein micelles and proposed that the α_{s1} - β - and κ -monomers were aggregated by calcium into small subunits in much the same fashion as Waugh et al. (69) had proposed for the entire micelle. Morr's subunits, as estimated by sedimentation velocity, have a diameter of about 30 nm. The subunits are stabilized by hydrophobic bonding

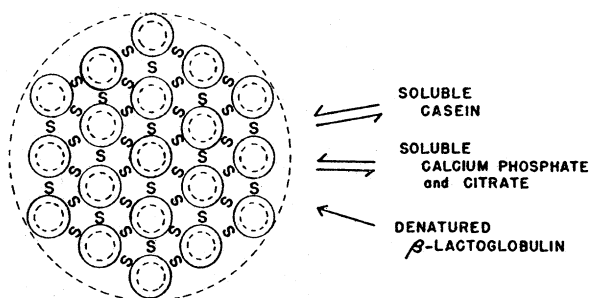


Fig. 5. Structure of the casein micelle after Morr (35). The S-shaped lines represent calcium phosphate linkages between small spherical complexes of the α_{s1} -, β - and κ -caseins.

and calcium caseinate bridges, and these subunits, in turn, are aggregated into micellar structure by colloidal calcium phosphate. Morr's model is summarized in Fig. 5. The average subunit size, postulated by Morr, is somewhat larger than that of Shimmin & Hill.

The hypothesis of Shimmin & Hill (52), that sections of the casein micelles contained particles of about 10 nm diameter, was invoked by Carroll et al. (8) and by Farrell & Thompson (17) who observed, by electron microscopy, particles of about 10 nm diameter in the Golgi vacuoles of lactating rat mammary gland. These particles were uniform in size and may be the precursors of thread-like structures which, in turn, coalesce into the spherically shaped casein micelle (Fig. 6 a, b). Others have reported similar observations in bovine mammary tissue. The biosynthesis of the casein micelle from small subunits was correlated with the disruption of casein micelles by dissociating agents by Carroll et al. (8). Using EDTA, urea, sodium lauryl sulfate, and sodium fluoride to disrupt micelles, the latter workers found particles of about 10 ± 2 nm diameter, and they noted that micelle assembly from subunits should lead to a rather uniform distribution of the α_{s1} -, β - and κ -caseins both on the surface and in the interior of the casein micelle. Schmidt & Buchheim (50) dialysed milk free of calcium in the cold and also used high pressure to disrupt casein micelles; in both cases, they obtained subunits of 10 nm diameter.

Subsequently, Pepper (40) has reported a Stokes radius of about 5 nm for the first cycle (Ca^{2+} free) casein as determined by gel filtration. The first cycle casein, after gel filtration, contained, qualitatively, all of the major casein fractions. The question yet to be resolved is whether or not the

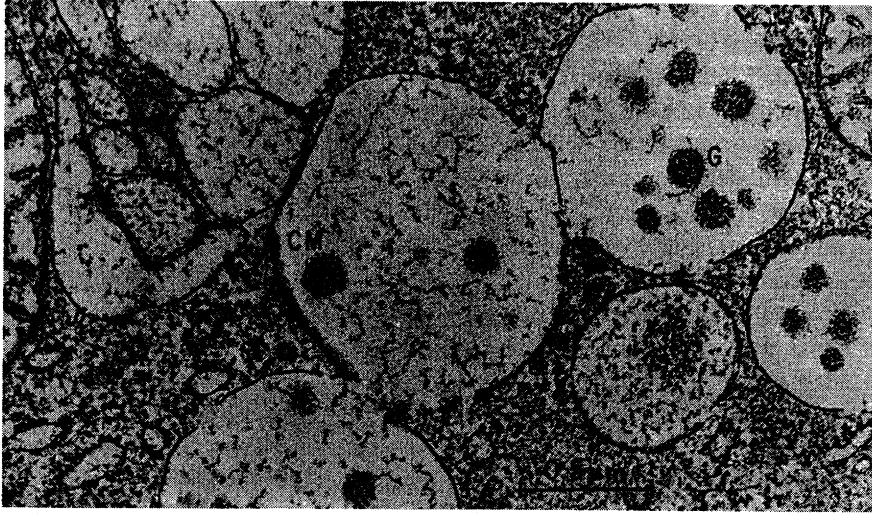


Fig. 6a. Formation of casein micelles (CM) within Golgi vacuoles (G) of lactating rat mammary gland. Initially, thread-like structure with some degree of periodicity appear, then more compact micelles seem to occur. Sections of the gland were fixed in buffered OsO_4 , Epon embedded, and stained with uranyl acetate and lead citrate; Carroll et al. (9).



Fig. 6b. A Golgi vacuole about to discharge its contents into the alveolar lumen. The Golgi vacuole shown appears to impinge upon the plasma membrane. A casein micelle is already present in the lumen; Carroll et al. (9).

casein subunits observed by all of the above workers exhibit any stoichiometry in terms of their α_{s1} -, β - and κ -casein content.

It has long been recognized that at least the α_{s1} - and κ -casein components occur in close association in the ' α -casein complex', with β -casein being more loosely connected to the micellar complex. Furthermore, the total micellar casein exhibits an overall ratio of 3 α_{s1} - : 2 β - : 1 κ -casein. The apparent uniformity of first cycle (Ca^{2+} free) casein and the subunits of the Golgi vacuoles would argue in favor of some consistent stoichiometry, but there exists the reported correlation between micelle size and κ -casein content which would argue against uniform subunit composition. Thus the existence of some type of subunit structure appears certain and the question to be decided now is the nature of these reported subunits. From the biosynthetic point of view, the build-up of the micelle from subunits is quite attractive, as it brings the casein components into the region of assembly with minimal interactions. Addition of calcium ions could cause the polymerization of casein subunits into longer chains and these chains could be stabilized into micellar spheres by the deposition of colloidal calcium phosphate. The assembly of the micelle from preformed subunits need not be as specific as that of tobacco mosaic virus, but the analogy is worthy of consideration. In the latter case, the structured RNA core of the virus plays a vital role in directing the correct particle assembly, whereas in the case of the micelle, only amorphous apatite could serve in this fashion. In attempting to solve the problem of casein micelle structure, it should be borne in mind that the biological function of the micelle is to provide an efficient nutritional fluid. Hence, the interactions which yield this product, the casein micelle, need not be as specific as those which result in the formation of a virus or an enzyme.

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